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14. ABSTRACT

The development of MDR in metastatic breast cancer is the primary cause of failure in the current chemotherapy treatment regimens. Although the precise nature of this clinical phenomenon is unclear and is likely due to several factors, the majority of breast tumors, like several other cancers, acquire resistance by multidrug efflux pumps. Several inhibitors (like Novartis compound PSC833) have already been discovered targeting both human MDR transporters. These MDR reversing agents however have been lacking in their potencies and their mechanism of action has been unknown. We have applied a highly integrated and innovative approach for the discovery of potent MDR inhibitors of breast cancer MDR by combining the most cutting-edge methods in chemical synthesis, drug discovery, and molecular structure. We have implemented click chemistry to produce a library of inhibitors, (2) developed functional assays, and (3) determined the structure of a close ortholog of hMDR1 with these anti-cancer compounds.

15. SUBJECT TERMS
Multidrug Resistance, Structure, transporters, click chemistry, functional assays

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Table of Contents

Introduction

The development of MDR in metastatic breast cancer is the primary cause of failure in the current chemotherapy treatment regimens. Although the precise nature of this clinical phenomenon is unclear and is likely due to several factors, the majority of breast tumors, like several other cancers, acquire resistance by multidrug efflux pumps. Several inhibitors (like Novartis compound PSC833) have already been discovered targeting both human MDR transporters. These MDR reversing agents however have been lacking in their potencies and their mechanism of action has been unknown. We have applied a highly integrated and innovative approach for the discovery of potent MDR inhibitors of breast cancer MDR by combining the most cutting-edge methods in chemical synthesis, drug discovery, and molecular structure. We have implemented a new style of chemistry called click chemistry to produce a library of inhibitors, (2) developed robust and high-throughput functional assays that are amenable to the high-throughput mode, and (3) determined the structure of a close ortholog of hMDR1 with these anti-cancer compounds, including the Novartis anti-cancer MDR reversal compound PSC833 that is in clinical trial.

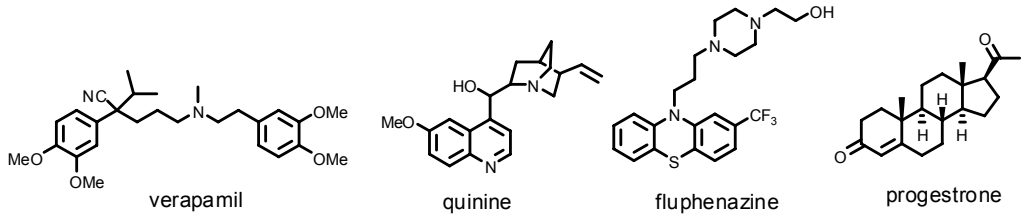
Body

Task 1. Synthesize potential inhibitor compounds using click chemistry against human MDR1

We have synthesized a variety of small molecules which are designed based on several criteria. First, since a large number of molecules are known to either bind to P-gp's or be transported by them, we have a good idea of the general features required for interaction with the target. Not surprisingly, they can all be classified generally as amphiphiles, most bearing positively charged groups and aromatic or aliphatic hydrophobic regions. Halogenated aromatic rings or heterocycles are common. A wide variety of diverse structures having these features were synthesized, employing the techniques of click chemistry as much as possible. The true value of such an approach is that lead compounds made by click chemistry are far easier to expand upon with rapid structure–activity correlations than are starting materials that are more expensive or difficult to obtain.

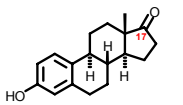
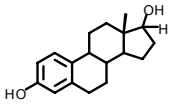
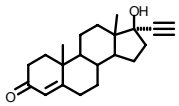
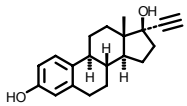
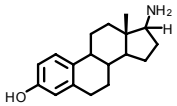
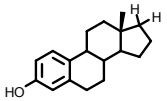
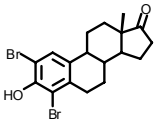
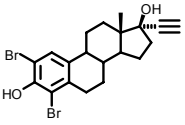
In addition, while we have widely searched for and manipulated compounds that would not be cytotoxic themselves but would inhibit MDR transporters, such as calcium channel blockers (eg. verapamil), antimalarials (eg. quinine), psychotropic-phoenothiazines (eg. fluphenazine) and steroid hormones (eg. progesterone), we found some interesting results from estrone derivatives, which is a type of steroid hormones, for MsbA (see Fig. 1). Estrone is known as an estrogenic hormone secreted by the ovary and a derivative of progesterone.

Figure 1: Starting compounds for chemical manipulation



As described in Table 1, estrone itself did not show any activity for MsbA but interestingly ethynylated estrone on C17 position (17α-ethynylestradiol, compound **3**) showed an inhibiting activity for MsbA. Thus, to determine which functional group in compound **3** is a crucial key for MDR reversal motion, we have focused on the manipulation of compound **3**. First, we were able to know that the functions of aromatic ring system (**2** vs **3**) and alkyl group on C17 (**1** vs **3**) play an important role for inhibiting MDR of MsbA. Instead of hydroxyl group on C17, amine substituted compound **4** shows a mild inhibiting activity without ethynyl group. Surprisingly reduced compound **5** from estrone through Wolff-Kishner reduction shows a very strong inhibiting activity and a brominated compound **7** inhibits MDR function for MsbA much stronger than compound **6** does.

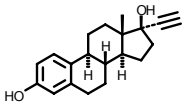
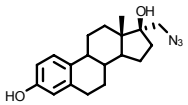
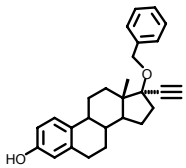
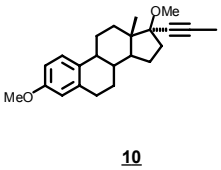
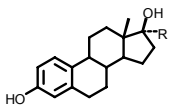
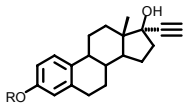
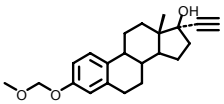
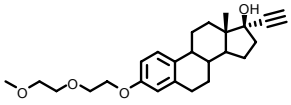
Table 1

compound (#batch)	activity for MsbA	compound (#batch)	activity for MsbA
 estrone	no activity	 **1**	no activity
 **2**	no activity	 **3**	inhibition (-44)
 **4**	inhibition (-20)	 **5**	inhibition (-81)
 **6**	inhibition (-47.3)	 **7**	inhibition (-77.9)

Inhibition and Activation measured by ATPase activity

Table 2 shows the different activity for MsbA depending on the different functional groups of compound 3. In place of ethynyl group (compound **3**), methyl, allyl or benzyl group enhanced (compound **11**) its inhibiting activity. Interestingly, compound **9** activated MDR function at the low concentration, and inhibited it at the high concentration. Instead of alkyl substitution of hydroxyl group on aromatic ring system (compound **12**), poly ethylene glycol substituted compound (compound **13** and **14**) shows much better MDR inhibiting activity for MsbA.

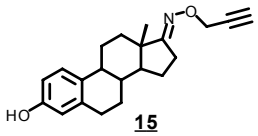
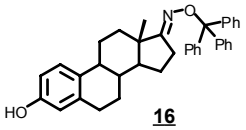
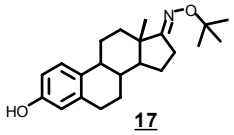
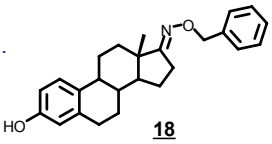
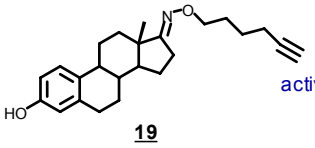
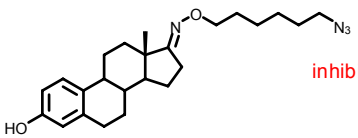
Table 2

compound (#batch)	activity for MsbA	compound (#batch)	activity for MsbA
 **3**	inhibition (-44)	 **8**	inhibition (-27)
 **9**	activiation (+36):L.C. inhibition (-61):H.C.	 **10**	activation (+42)
 **11**	R: methyl, inhibition (-63) allyl, inhibition (-68) benzyl, inhibition (-68)	 **12**	R: methyl, inhibition (-28) benzyl, inhibition (-27)
 **13**	inhibition (-71)	 **14**	inhibition (-82)

Inhibition and Activation measured by ATPase activity

We generated oxime derivatives of estrone due to its hydrogen bonding ability as described in Table 3. Most of them are activators for MsbA but interestingly compound **17** plays a role of activator at low concentration and inhibitor at high concentration.

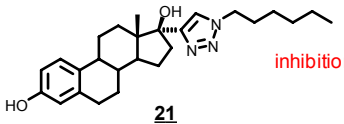
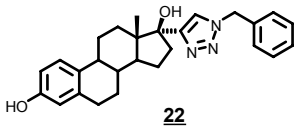
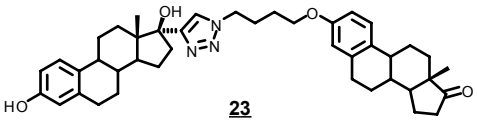
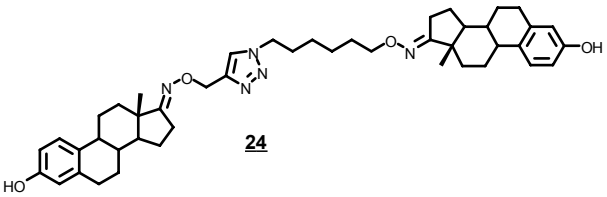
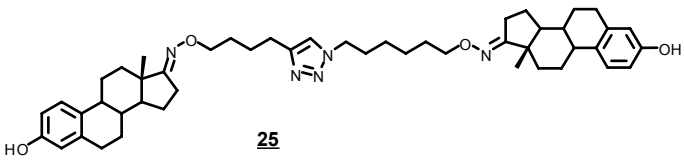
Table 3

compound (#batch)	activity for MsbA	compound (#batch)	activity for MsbA
 **15**	activation (+29)	 **16**	activation (+36)
 **17**	activation (+44): L.C. inhibition (-70): H.C.	 **18**	activation (+30)
 **19**	activation (+58)	 **20**	inhibition (-31)

Inhibition and Activation measured by ATPase activity

Table 4 shows some clicked compounds of estrone derivatives.

Table 4

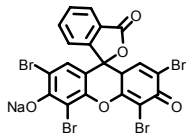
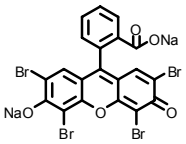
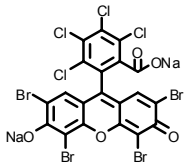
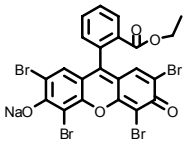
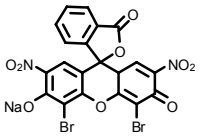
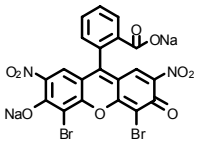
compound (#batch)	activity for MsbA	compound (#batch)	activity for MsbA
 **21**	inhibition (-59.9)	 **22**	inhibition (-52.4)
 **23**	inhibition (-27.1)		
 **24**	inhibition (-30)		
 **25**	activiation (+20)		

Inhibition and Activation measured by ATPase activity

We have also found an interesting result from eosin family. Eosin is an remarkable bright red dye that benefits a myriad of applications with the effectiveness of its intense color (eg. it is used in biology to stain cells). As shown in Table 5, ethyl eosin (compound **29**) shows the best inhibiting activity for MsbA. When compared with

compound **26**, acyclic ester backbone of compound **29** is more important key than lactone ring system of compound **26** for MDR reversal motion. Also chlorinated aromatic ring of eosin Y (phloxin B, compound **28**) shows much better inhibitor than non-chlorinated compound **27** eosin Y). Nitro functional group on eosin (compound **30** and **31**) plays no role to inhibit MDR for MsbA.

Table 5

compound (#batch)	activity for MsbA	compound (#batch)	activity for MsbA
 Eosin Y, spirit soluble (**26**)	activation (21)	 Eosin Y (**27**)	activation (19)
 Phloxine B (**28**)	inhibition (-78)	 Ethyl Eosin (**29**)	inhibition (-88)
 Eosin B, spirit soluble (**30**)	no activity	 Eosin B (**31**)	no activity

Inhibition and Activation measured by ATPase activity

Task 2. Modification known inhibitors of human MDR1 and ABCG2

Application of click chemistry for drug discovery and lead identification for MDR inhibitors. We have improved the potency of inhibitors of MDR ABC transporters using a modular approach applying click chemistry to facilitate not only lead identification but also their optimization. We will initially use cancer compounds that bind MsbA targeting the "ON" and "OFF" sites to tie the inhibitors/substrates together to jam MsbA specifically and with high potency. The click chemistry approach to drug discovery enables us to use a set of extremely reliable and modular organic transformations (Kolb, Finn, and Sharpless et al., 2001; Kolb and Sharpless, 2003). Click chemistry can be used in a "traditional" sense for rapidly assembling combinatorial libraries of drug-like compounds for screening, or in the new *in situ* mode (Lewis et al., 2002; Bourne, 2004; Manetsch et al., 2004), in which the target assembles its own inhibitors. Both approaches have been successfully used in our labs for lead discovery and optimization (Kolb et al., 2000; Lewis et al., 2002; Bourne et al., 2004; Lee et al., 2003). Click chemistry serves as a guiding principle in the quest for function: the search must be restricted to molecules that are easy to make. Focusing on "lead" discovery, this strategy provides a means for the rapid exploration of the chemical universe. For "lead" optimization, it enables rapid structure-activity profiling through generation of analog libraries. Click chemistry does not replace existing methods for drug discovery, but rather it complements and extends them. It works well in conjunction with structure-based design and combinatorial chemistry techniques, and, through the choice of appropriate building blocks, can provide derivatives or mimics of 'traditional' pharmacophores, drugs and natural products (Kolb et al; 2001; Sneader, 1996; Bemis, 1996). However, its real power lies in its ability to discover leads that may not necessarily resemble known pharmacophores.

Click chemistry utilizes modular heteroatom-bond forming reactions that approach perfection, by virtue of having a high driving force, being reliable, selective, and easy to perform and work up (Fig. 25). It employs many condensation reactions, such as the formation of oxime ethers and heteroaromatic systems, as well as

nucleophilic substitution reactions on strained compounds and intermediates. The 1,3-dipolar cycloaddition of alkynes and azides yielding triazoles is one of the premier example of a click chemistry reaction (Huisgen, 1984). Azides and alkynes are highly energetic functional groups that are easily introduced into organic compounds, yet they are inert to a variety of reagents and to biological systems. A significant breakthrough was the recent discovery of a copper(I)-catalyzed, step-wise-variant of Huisgen's concerted cycloaddition process, which proceeds under very mild conditions and is accelerated by 10^8 over the parent thermal [2+3] cycloaddition (Rostovtsev et al., 2002; Wang et al., 2003; Tornoe, 2002). There are no protecting groups, and with near complete conversion and selectivity for the 1,4-disubstituted triazole, yield and structural uncertainties don't exist, and purification requirements are minimal. The transformation is especially relevant for drug discovery, due to the favorable physicochemical properties of triazoles, which are proving to be potent pharmacophores in their own right. They possess a large dipole moment of ~5 Debye (N-methyl acetamide: 3.7 – 4.0 Debye; Purcell and Singer; 1967), and nitrogen atoms 2 and 3 function as weak hydrogen bond acceptors.

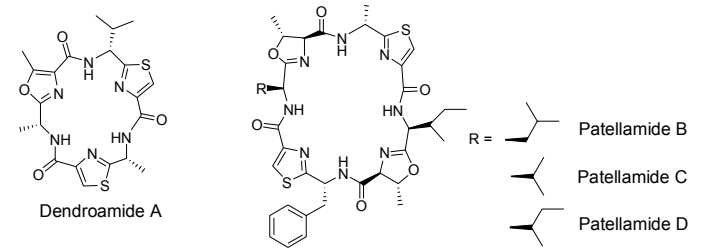


Figure 2. Natural cyclic peptides with MDR reversing activity

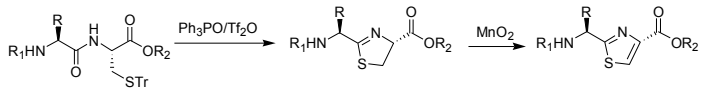


Figure 3. Synthesis of thiazolines and thiazoles from cysteine-containing peptides using Ph_3PO/Tf_2O.

Studies with cyclic inhibitors based on cyclosporin A and other MDR cyclo-peptides. Another exciting direction that we are exploring includes the investigation of cyclic peptides or peptidomimetics. The cyclic peptide valspodar (PSC833), a derivative of cycloporin A, may function as an effective MsbA modulator as it binds to and co-crystallizes with MsbA. However, further elaboration on this template is labor- and time-intensive because of the large ring size of this molecule (33-membered ring consisting of 11 peptides) and its structural complexity (Fig. 24). Several other families of cyclic peptides with relative simpler structures have been shown to be able to reverse MDR by binding to Pgp. The natural products dendroamide A and patellamides B, C and D are the main representatives of this group (Fig. 2) (Fu et al., 1998; Ogino et al., 1996; Williams and Jacobs, 1993). These molecules typically have a smaller ring size than cycloporin A derivatives (hexa- or octapeptides) and exhibit thiazole, thiazoline, oxazole or oxazoline structural units. Less attention has been attributed to modify these structures in order to increase their potency as MDR inhibitors in contrast to the tremendous work which has been dedicated to the synthesis of cycloporin A derivatives. The recent work in the laboratory of Dr. Jeffery Kelly (TSRI) has highlighted the synthesis of these classes of molecules. Dr. Zhang, the co-PI leading the chemistry effort in this proposal, was trained as a post-doctoral research associate in the Kelly laboratory and is very familiar with these reactions. The chemistry for oxazole and oxazoline synthesis has been well established in the literature, whereas synthesis of thiazole and thiazoline in peptides appears to be problematic. The discovery of a P(V) reagent (hexaphenyl-oxodiphosphonium trifluoromethanesulfonate) allows a very facile and efficient synthesis of thiazole and thiazoline directly from protected cysteine residues (Fig. 3) (You et al., 2003). This newly developed synthesis pathway has led to the efficient synthesis of a number of cyclic peptides composed of thiazole structures including the P-gp inhibitor dendroamide A (Fig. 4) (You et al., 2003; You and Kelly, 2003,2004). Furthermore, applying this new methodology into solid-phase synthesis makes it possible to quickly access a large number of target molecules (You and Kelly, 2003 & 2005), thereby enhancing chances of finding more potent inhibitors.

Lipophilicity has been demonstrated in many cases to be a very important parameter in MDR inhibitor design which is not surprising because the drug must cross the cell membrane to interact with the target proteins (Klopman et al., 1997). Actually, comparison of dendroamide and patellamide series reveals that the P-gp inhibitors among them appear to have a higher lipophilicity than those analogues without inhibitory activity (Fu et al., 1998; Oginio et al., 1996; Williams and Jacobs, 1993). This observation suggests that increasing the

lipophilicity of these cyclic peptides could have a positive effect on inhibiting MDR transporters including MsbA. This could be accomplished by incorporating more hydrophobic amino acids (Ile, Leu, Val and Phe) into the sequence, and/or by masking the amide N-H with N-methyl group as in cycloporin A derivatives. A benefit using N-methylated peptides is their resistance to proteolysis *in vivo* and improved oral bioavailability.

Another variant to these structures includes the cyclodepsipeptides. Several cyclodepsipeptides including the analogues of cyclosporine have been demonstrated to exhibit relatively high chemo-sensitizing potency (Kurome et al., 1998; Kurome, Takesako et al., 1998; Loor et al., 1992; Stratmann et al., 1994). The chiral α-hydroxy acid equivalents of the respective α-amino acids are readily available and can be incorporated accordingly into solid-phase synthesis using the *t*-Boc strategy (Deechongkit et al., 2004). Many other commercially available chiral α- or β-hydroxy acids with hydrophobic branches will be explored as well during the synthesis of the library. Furthermore, a variation of click chemistry (Section D3.2) can also be applied in the cyclic peptide synthesis, which has been confirmed in a head-to-tail peptide dimerization process (Punna et al., 2005). The heterocyclic triazole structure is mimetic to those of thiazole and oxazole, and it could be unique in the MsbA inhibitor design. We would prefer to use a click reaction in monomer cyclization instead of dimerization, and it is yet to be investigated and optimized in our synthesis. The additional advantage of using click chemistry may be the high efficiency in the final cyclization step which often results in the overall low yield of cyclic peptide synthesis. We intend to design and synthesize cyclic peptides or peptidomimetics with a smaller ring size (similar to that of dendroamides and patellamides) relative to cycloporin A derivatives, which will facilitate the synthesis of a library and render its synthesis more practical. Adjusting the ring size, varying the number of thiazole, thiazoline, oxazole or oxazoline cycles, and incorporating unnatural and different configurations of amino acids or hydroxyl acids will add additional flexibility to our design and synthesis. We have demonstrated that several of these compounds have transport and ATPase profiles similar to PSC833 (data soon to be published).

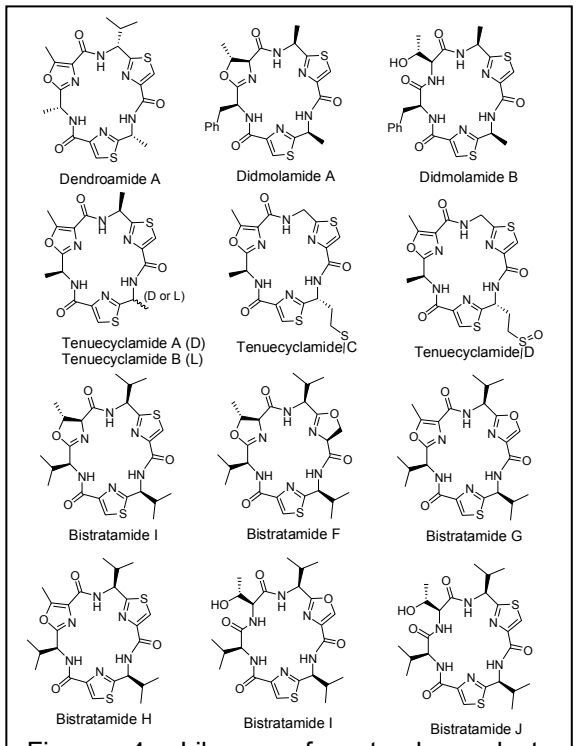


Figure 4. Library of natural products composed of oxazole and thiazole structures synthesized in Kelly laboratory. These compounds are chemically simpler compounds of PSC833 and some have already been tested for ATPase inhibition of MsbA. Those that demonstrate binding to MsbA (Section D2) will be used for co-crystallization (Section D4).

Task 3. Modification of known modulators of estrogen receptors

We have discontinued this task to focus more on the inhibitors/modulators of MDR pumps taking the suggestion of the reviewer of the grant.

Task 4. Over-produce human MDR1 and ABCG2

Sub-Contract Report on MDR1 and ABCG2 expression: Ina Urbatsch (TTUHSC)

1) Production of purified P-glycoprotein for crystallizations and inhibitor screening. We have grown 26 fermentor cultures of various mouse and human P-glycoprotein (Pgp) wild-type and mutant strains and have harvested ~46 kg of cells. So far more than 200 batches of cells (100 g each) were processed resulting in 910 mg of purified Pgp (35 purifications) that reached the crystallization trials. Unfortunately, formation of crystals has been variable and diffraction quality crystals have not been obtained. We realize that the detergent concentration in the final purified material was high and variable and likely interfered with the formation of

highly ordered crystals. We subsequently screened different resins that bind Pgp with high affinity and allow for removal of the detergent on the column before final concentration of the protein on Amicon filters. For this we also constructed a Pgp variant with a double affinity tag consisting of a calmodulin binding peptide and a His_6-tag. Unfortunately, the larger tag at the C-terminus caused degradation of Pgp. We continue to exploring different combinations of affinity tags at the N-terminus to improve the current purification procedure.

After a visit of Geoffrey Chang, Andrew Ward, and Jodie Yu to my lab last November and extensive discussions we decided to move the purifications to Geoffrey' lab in order for the proteins to reach the crystallization trays without freezing of the purified material. We have since scaled up on membrane preparations and have already provided 85 batches of membranes (each from 100 g of cells) for purifications. Within a short period of time the Chang group has adopted our purification scheme and indeed significantly improved the materials by shortening the purification into a 6-7 hour procedure. They are now purifying highly active Pgp in low detergent concentrations and I am very confident that this improved material will lead to diffraction quality crystals.

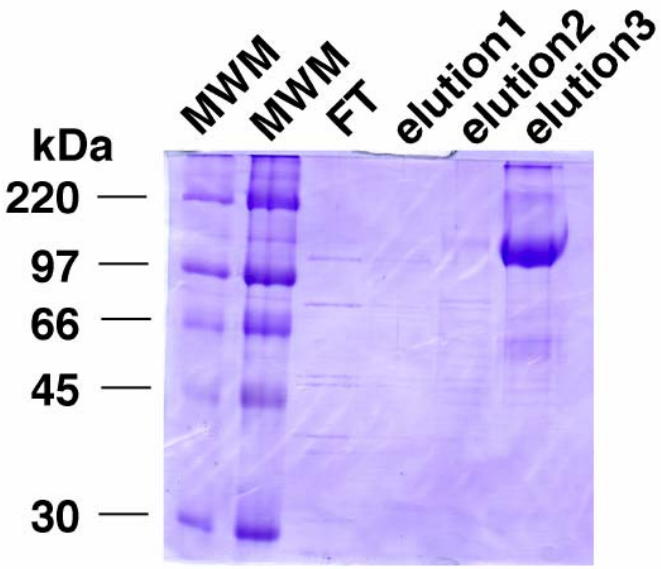


Figure 5. Commassie stain gel of MDR3. Lanes (left to right): Markers (2), Flow through, Three elution lanes (last lane containing the purified MDR3 protein).

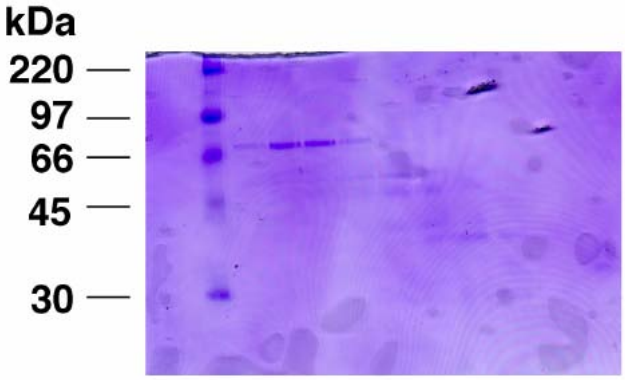


Figure 6. Commassie stain gel of ABCG2 purification.

2) Purification of ABCG2 (Breast Cancer Resistance Protein) and members of the MRP family. We have commenced working with Steve Aller, a new post doctoral fellow in the Chang group, in fine-tuning the purification of ABCG2. This half-size ABC transporter has been suggested to function as homo-dimer as well as higher oligomer species and therefore poses a challenge to purifying in a homogeneous and functionally active form that is suitable for crystallizations.

3) Extended projects: We previously developed a *P. pastoris* vector for the high-throughput cloning and expression screening of human ABC transporters. Here we will generate different versions of this vector containing N-terminal affinity tags to screen for high-level expression of a variety of mammalian Pgp orthologs and thereby increase the probability of obtaining crystals with the highest possible resolution.

In summary we have made much progress and are confident that our continuous efforts in close collaboration with the Chang and Finn groups will lead to one or more crystal structures of these multidrug transporters and the discovery of more potent inhibitors that will reverse breast cancer multidrug resistance.

<u>Chang Lab results on MDR3/MDR1 and ABCG2</u>

We have made significant progress in the production of both MDR3/MDR1 and ABCG2. We have obtained very high yields of MDR3/MDR1 with very good purity suitable for x-ray crystallography and functional

analysis (Fig. 5). We have further improved the purification procedure for Ina to accomplish this task in less than 10 hours. We have also made significantly progress on purifying human ABCG2, which is involved in breast cancer multidrug resistance (Fig. 6). Although the protein preparation of ABCG2 is a bit more complicated than MDR3, we are making progress streamlining the large-scale preparation. We believe that we should have ABCG2 purified in both the protein yield and purity suitable for functional and structural studies by x-ray crystallography.

Task 5. Over-expression and purification of human estrogen receptor

We have discontinued this task to focus more on the inhibitors/modulators of MDR pumps taking the suggestion of the reviewer of the grant.

Task 6. Assay function of estrogen receptors

We have discontinued this task to focus more on the inhibitors/modulators of MDR pumps taking the suggestion of the reviewer of the grant.

Task 7. Functional assays of drug compounds with Pgp

Sub-Contract Report on Screening of Pgp inhibitors: Ina Urbatsch (TTUHSC)

So far, we have screened four batches of compounds (total of 136) that have been synthesized by the Finn group. These compounds were selected and designed for their inhibitory action on Pgp. While the first batches were clearly substrates of Pgp and stimulated the ATPase to different degrees some of the more recent developed compounds show increasing potential of inhibiting Pgp function. In addition we have commenced screening the NIH repositories (~140,000 compounds) with funding provided by the Wilson Foundation, Dallas. We have identified several compounds that inhibit Pgp at moderate concentrations and have communicated the chemical structures to the Finn group for integration into the lead optimization. All inhibitory compounds are currently in crystallization trials (Chang group) and we have commenced the functional analysis of the hit compounds to dissect their mechanism of action.

Chang Lab Report

We have also screened hundreds of synthesized compounds for their effect on function of the ABC transporter MsbA. This investigation serves three purposes: (1) develop novel inhibitors of this essential protein for use as antibiotics, (2) elucidate the mechanism of action in ABC transporters by exploring the structure/activity relationship of substrates that interact with MsbA and Pgp and (3) develop brominated/mercurated derivatives of these compounds to use for analysis by x-ray diffraction of co-crystals with MsbA, structurally defining binding pockets. Both MsbA and Pgp (along with other ABCs) have been shown to interact with a wide spectrum of structurally distinct molecules, yet the mechanism by which the protein accomplishes this poly-specificity is not well understood. We are attempting to probe this multivalent nature by screening libraries of compounds.

To date we have developed four assays to test effect on activity: (1) a linked enzyme ATPase assay, (2) a doxorubicin efflux assay, (3) a direct efflux of substrate (estrones) assay and (4) a bacterial kill assay. An example of the use of these assays on one compound is shown in Fig. 7. The ATPase assay measures the rate of ATP hydrolysis in the presence of increasing amounts of compound being tested. From this we can learn if the compound stimulates, inhibits, or has no effect on the ATP turnover in MsbA. This is important because ATP hydrolysis drives transport of substrate. Targeting ATPase activity is one way to shutting down the transporter. We have characterized ~200 different compounds based on ATPase activity. The doxorubicin efflux assay measures the rate of doxorubicin removal from the cell in the presence of the compound being tested. Here we are looking to find compounds that block transport. Transport can be inhibited by shutting down ATPase as above or by competing with substrate (doxorubicin) for binding and transport. The direct efflux assay will measure the direct transport of the estrone family of compounds that we are exploring for their antimicrobial activity. Lastly the bacterial kill assay measures the dose needed to inhibit bacterial growth.

All four assays are up and running, with some tweaking of the direct efflux assay in the works. We started with the ATPase assay and identified ~20 compounds that have significant effect on activity. Effects range from 90% inhibition to 60% stimulation of ATPase. Out of the 20 we have focused on 2 classes of compounds, estrones and the eosins. We have synthesized ~40 estrone derivatives and ~10 eosin derivatives for testing. While most members of these classes inhibit ATPase there are representatives from each class that stimulate ATPase. Interestingly, ATPase stimulation/inhibition are not directly correlated with transport activity as tested by the doxorubicin efflux assay. Also, some compounds show no effect on ATPase activity, yet inhibit transport. We see up to ~45% transport inhibition of doxorubicin efflux. Preliminary kill assay data show a decrease in growth of bacterial colonies exposed to some compounds, but further testing needs to be done.

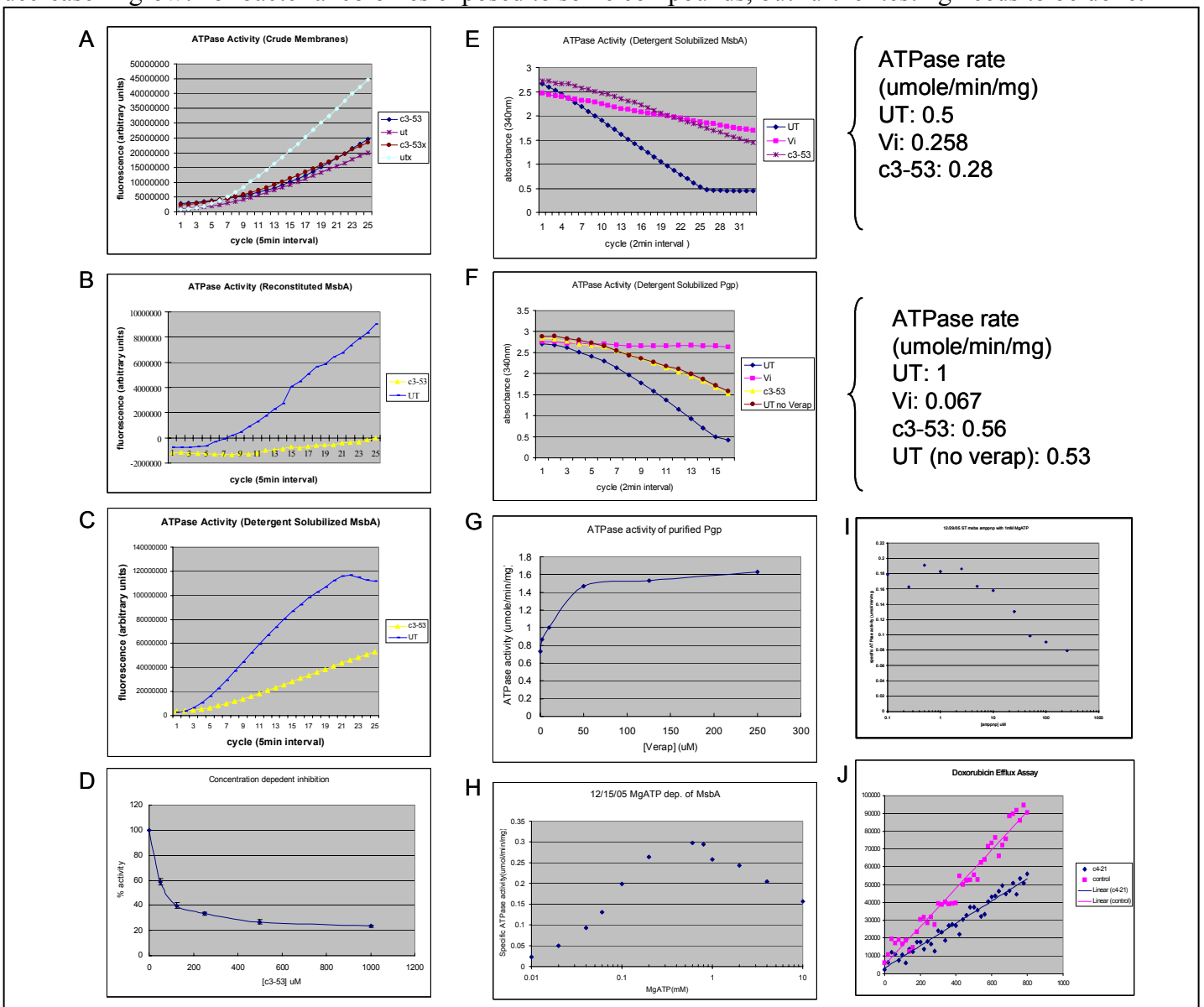


Figure 7. ATPase Activity Assays (PiPer: A-D; Linked Enzyme: E-G; Malachite Green: H-I) and doxorubicin transport assay (J). Msba (a MDR ABC transporter of Pgp) overexpressing crude membranes (utx) are ~2x active compared to control membranes (ut) (A). Compound c3-53 (250uM; chemical structure not shown) knocks ATPase activity of MsbA overexpressing membranes (c3-53) down to control membrane level (c3-53) (A). Compound c3-53 also shows inhibition of reconstituted and detergent solubilized MsbA (B-C). (D) Concentration dependent inhibition of MsbA by c3-53 (D). (E) Inhibition of MsbA by c3-53 and vanadate shown in LE assay. (F) Inhibition of Pgp by vanadate and c3-53. (G) Verapamil stimulated ATPase activity of Pgp. (H) Specific Activity of MsbA with increasing concentrations of MgATP. (I) Concentration dependent inhibition of ATPase activity with increasing concentrations of the non-hydrolyzable ATP analogue, AMP-PNP. (J) Doxorubicin efflux assay demonstrating inhibition of MsbA using compound C4-21.

Task 8. Determine high-resolution x-ray structure of human MDR1 and ABCG2

Attacking the ON and OFF site of MDR ABC transporters (a structural approach)

Our first focus was to ascertain if our compounds could be co-crystallized. We chose as a model system MsbA because of it's close protein sequence and structural homology to human MDR1. The availability of these structures to study the "ON" and "OFF" sites is a major advantage in the design of compounds. The x-ray structures of the uncomplexed forms of MsbA from *E. coli* (4.5 Å; Chang and Roth, 2001; MsbA-EC; Fig. 8a) and *V. cholera* (3.8 Å; Chang, 2003; MsbA-VC Fig. 8b) have revealed the overall structural topologies of this lipid flippase and also demonstrates the dynamic nature of the transporter. The structures reveal that MsbA can adopt a scissor like motion in the transmembrane portion of the molecule and that the nucleotide binding domains (NBD) can have an ensemble of conformations in the absence of ATP or nucleotide analog. The recent structure of MsbA from *S. typhimirum* (MsbA-ST; Fig 8c) provides structural insights into the LPS binding sites and the transport mechanics for lipid flippases and MDR ABC transporters (Reyes and Chang, 2005). This structure is in complex with Mg^{2+}, ADP, vanadate, and the rough-chemotype lipopolysaccharide, Ra LPS. The structures supports a model involving a rigid-body torque of two transmembrane domains (TMD) during ATP hydrolysis and suggests a mechanism by which the NBDs communicates with the TMDs. Together with the previously observed MsbA conformations, this new structure strongly suggests a "flip-flop" mechanism in which the sugar groups are sequestered in the chamber while the hydrophobic tails are dragged through the lipid bilayer.

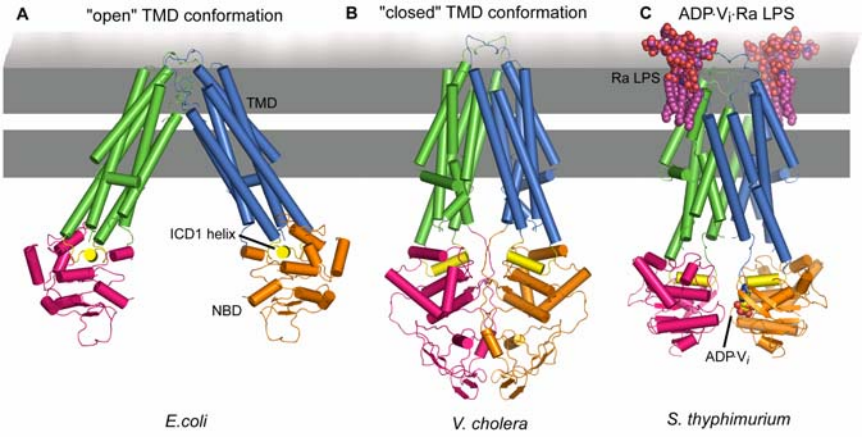


Figure 8. Three x-ray structures of MsbA showing conformational changes. (A) Open conformation from *E. coli*, (B) Closed conformation from *V. cholera*, (C) Post-hydrolysis conformation trapped with Ra LPS and also ADP+vanadate from *S. typhimirium*. The cell membrane is shown in gray. The Nucleotide binding domains (NBD) are shown in orange and violet. Structures suggest that MsbA can adopt a wide range of conformations to transport a LPS, a large amphipathic substrate.

Structural studies of MsbA concerning substrate translocation. The structure of ST-MsbA complexed to LPS and trapped with ADP•vanadate reveals a structural basis for substrate translocation coupled to ATP hydrolysis. Changes in the TMD interactions provide insight into the pathway of substrate efflux. In comparison with the apo closed conformation of MsbA, the ST-MsbA structure complexed with LPS exhibits a large rigid body rotation and translocation that results in a ~15 Å opening toward the periplasmic ends and a ~15 Å closing of the NBD-associated intracellular domain helices (ICD1; residues 111 to 121), which allow accessibility to an internal chamber from the periplasm but from the cytoplasm (Fig. 9). The third extracellular loops (EC3) mediate the internal contact between the two monomers while placing the periplasmic ends of the TM5 helices close together. This causes the periplasmic opening of the internal chamber to be pinched and divides the opening of the cavity into two lobes adjacent to TM6, which corresponds to drug binding sites observed to LmrA and human Pgp. In this structure, TM5 forms extensive intermolecular interactions with TM2 and TM3. Because the ICD1 is formed between TM2 and TM3, this interaction suggests a probable pathway for transmitting conformational changes caused by ATP-hydrolysis to the substrate binding sites. Besides affecting the interactions between TMDs, substrate binding and ATP hydrolysis also drive changes in the intermolecular helical packing of the TMDs. Although the TM1 and TM4 helices are arranged in an overall architecture similar to both conformations, TM5 and TM6 reveal significant rearrangements [Root mean square deviation on Ca of 2.1 Å (open) and 1.9 Å (closed)] (Fig 10).

Structural studies concerning substrate recognition by MsbA. Our initial binding data derived from functional experiments (analytical gel filtration followed by electro-spray mass spectrometry; data not shown) and direct crystallographic measurements using several hydrophobic cationic heavy atom compounds have revealed that MsbA has two substrate binding sites (called "ON" and "OFF") for lipid (and also possibly other hydrophobic cationic compounds like cancer drugs) located near an elbow region composed mostly of transmembrane helices TM1, TM5, and TM6 (Fig. 9). The "ON" site is located on the inner membrane leaflet side and is poised to accept lipid A from the inner membrane leaflet side. The "OFF" site is located on the outer membrane leaflet side where we observe the lipid A bound as it is existing the lipid bilayer. Both of these binding sites change from a "HIGH" to a "LOW" affinity during the course of the transport cycle and possibly caused movements in TM5 and TM6 (see Fig. 9). The structure of MsbA-ST reveals that the Ra LPS trapped in the "HIGH" affinity, "OFF" site just prior to resetting the transporter. The binding of lipid A at this position is facilitated by ADP and vanadate, which occupies the probable position of the γ-phosphate of the nucleotide. The proximity of these two drug binding sites on the outer face of the molecule provides an opportunity to design potent inhibitors to jam MsbA.

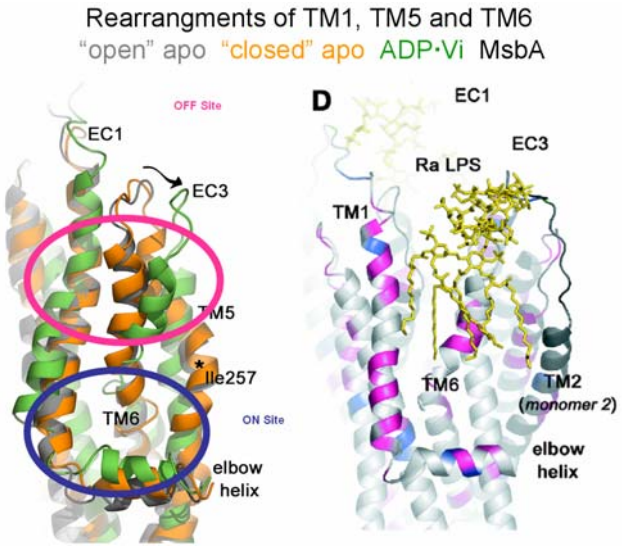


Figure 9. "ON" and "OFF" LPS binding sites of MsbA. **(Left)** Superimposed TMDs from open apo (grey), closed apo (orange) and post-hydrolysis conformation of MsbA (green) show the movement of TM5, TM6, and EC3 (as indicated by arrow). The helical bulge near residue Ile257 is indicated by an (*) **(Right)** Position of conserved residues shared by MsbA and Pgp (pink) and conserved residues specific to the MsbA subfamily (blue) mapped onto the structure of MsbA (white). The LPS is shown in yellow. The "ON" and "OFF" sites are indicated in blue and pink, respectively. The "OFF" site is in the high-affinity state.

Our MsbA preparation is a highly active ATPase. We have checked the ATPase activity of our preparations of MsbA in collaboration with Dr. Ina Urbatsch (TTUHSC) and have functional data indicating that the material used for crystallization has very high ATPase activity that is linear over time and independent of protein concentration (Fig. 10A). The material is also very robust, with less than 25% loss of ATPase activity upon freeze-thaw, which is consistent also with the crystallization results that we have previously observed. The size of the LPS head group also affects the stimulation of ATPase activity in MsbA. The degree of ATPase activity upon the interaction of various Lipid A-related moieties and is related to to the size of oligosaccharide domain of the LPS ("Ra" and "Re" LPS sugar head-group being the largest and smallest, respectively with the gradation in between). Interestingly, there is significant level of ATPase stimulation by rhodamine and Hoeschst, which are both extruded by human MDR1.

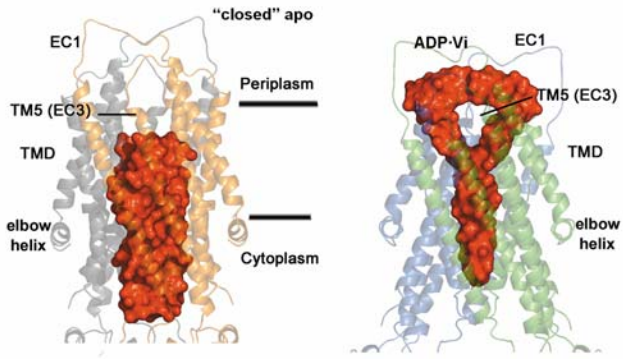


Figure 10. Structural basis for LPS translocation across the cell membrane caused by rearrangements in the TMD.(A) solvent-filled internal chamber (red) for the closed apo structure of MsbA (monomers in orange and gray). (B) Solvent-filled internal chamber for MsbA complexed with LPS and ADP•VO_3 (monomers in green and blue).

X-ray structure of PSC833 MsbA: a model for cancer chemotherapy MDR reversal

PSC-833 is a non-immunosuppressive cyclosporine derivative identified as a clinically effective chemosensitizer of multidrug resistance associated with cancer and leukemia[1]. For more than a decade PSC-833 has been utilized as a high affinity inhibitor of the ATP-binding cassette (ABC) transporter P-glycoprotein, yet the binding mode and structural mechanism of inhibition is not well understood[2]. Here we report the X-ray structure of MsbA, an ABC transporter with sequence and functional homology to P-glycoprotein, in complex with PSC-833 and ADP·vanadate. PSC-833 binds near the nucleotide binding domain and intracellular domain interface, conserved in both P-gp and MsbA. PSC-833 decouples ATP hydrolysis from substrate binding and closes the transmembrane translocation pathways. Our results reveal the molecular basis of inhibition in this class of ABC transporters. Understanding the structural and functional binding modes of PSC-833 will aid in the development of new therapeutics targeting ABC transporter mediated MDR in bacteria and human cancers.

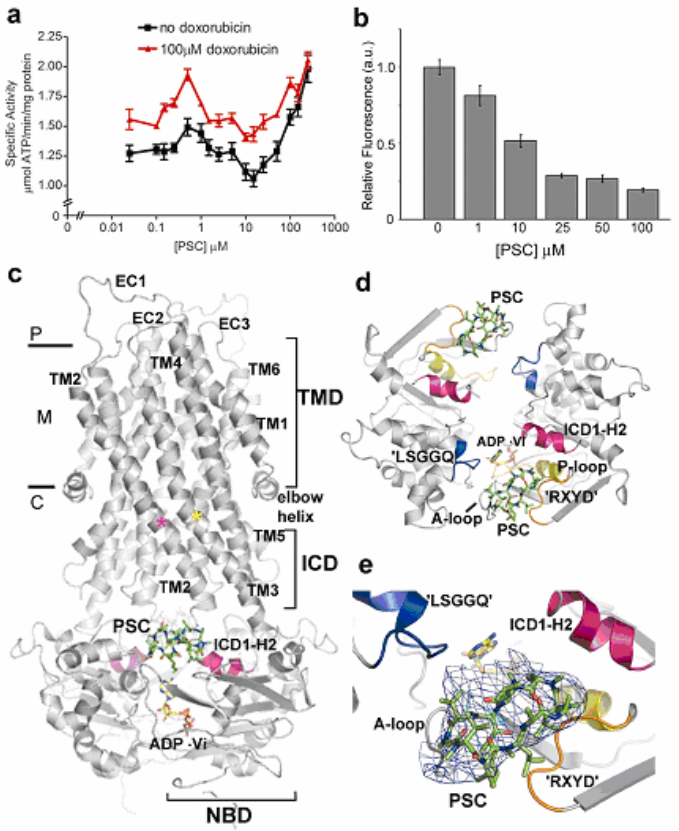


Figure 11. Functional and structural effects of PSC-833 on MsbA. **a.** Concentration dependent effect of PSC-833 (PSC) on untreated and doxorubicin stimulated ATPase activity in MsbA. **b.** Concentration dependent inhibition of doxorubicin efflux by PSC in Δ*acrAB E. coli* cells overexpressing MsbA ($P < 0.05$). **c.** Side view of MsbA homodimer (gray). The transmembrane domain (TMD) spans the lipid bilayer matrix (M), with the intracellular domain (ICD) and the nucleotide binding domain (NBD) within the cytoplasm (C). TMD helices are labeled (TM1-6). Two PSC molecules (carbon-green, oxygen-red, nitrogen-blue) are bound at the ICD1/NBD interface and ADP·vanadate (carbon-yellow, oxygen-red, nitrogen-blue, phosphate-purple, vanadate-orange) is bound to the ATP active site in one monomer. Highlighted in magenta and yellow (∗) are two conserved glycines (Gly141 and Gly94 respectively) in TM2 and TM3 located at the TMD/ICD interface. **d.** Top view of interacting NBDs down two-fold axis. PSC molecules are bound to each NBD near the A-loop (cyan), the 'RXYD' loop (orange), ICD1-H2 (magenta) and the 'LSGGQ' motif (blue). **e.** Electron density map (1.0σ) corresponding to bound PSC.

Resistance of cancer cells to chemotherapeutic drugs is largely mediated by the overexpression of multidrug transporters such as P-glycoprotein (P-gp, MDR1, ABCB1), an ATP Binding Cassette (ABC) transporter with functional homology to a sub-class of lipid flippases and drug transporters found in both prokaryotes and eukaryotes[3-5]. PSC-833 (PSC) is an industry standard for multidrug resistance (MDR) modulation; it is a highly specific and potent reversing agent of P-gp mediated drug efflux in acute myeloid leukemia, multiple myeloma, and ovarian cancer[1,2]. Biochemical studies of P-gp with PSC have suggested a unique mechanism of transport inhibition that results in disruptions of both drug efflux and modulation of ATP hydrolysis[6-9]. MsbA is one of several prokaryotic ABC transporters that has significant sequence homology to P-gp (>30% identity) and interacts with an overlapping spectrum of chemotherapeutic drugs both *in vivo* and *in vitro*[10-13]. The high protein sequence conservation between MsbA and P-gp along with functional homology supports a common structural architecture and mechanism[14]. Here, we report the functional characterization and X-ray structure of MsbA from *Salmonella typhimurium* in complex with PSC, which reveals unique insights into the biology of drug efflux and the mechanism of transport inhibition.

PSC modulates both the ATPase activity and transport activities of purified, of MsbA. ATPase activity of detergent solubilized MsbA[15] was measured using a linked enzyme assay[16,17] and shows a basal activity of 1.1-1.3 μmol ATP/min/mg of protein. This activity is equivalent to basal activity of P-gp that has been reconstituted in lipids[18] and is ~30-500 times greater than that previously reported for either detergent solubilized or lipid-reconstituted MsbA (Fig. 11a)[19,13]. P-gp ATPase activity exhibits a concentration dependent inhibitory effect within moderate concentration ranges of PSC (1 – 100 μM)[20,21]. In this study, ATPase activity in MsbA is modulated in a multimodal manner by PSC over a large concentration range: (*i*) at low concentrations (~150 nM) PSC stimulates ATPase 25% over basal level; (*ii*) at intermediate concentrations (~10 μM), PSC inhibits ATPase 15% under basal level; (*iii*) and at high concentrations (50 – 250 μM), PSC stimulates ATPase up to 55% over basal activity (Fig. 11a). This ATPase profile was comparable using different orthologs of MsbA and was confirmed with a separate ATPase assay directly measuring inorganic phosphate release (data not shown). Doxorubicin (100 μM) stimulates ATPase 25% over basal level (Fig 1a). PSC modulates doxorubicin stimulated ATPase activity of MsbA in a similar manner as untreated (Fig.1a). Transport inhibition of MsbA by PSC was evaluated by measuring doxorubicin efflux over time in an *E. coli* strain overexpressing MsbA. PSC decreases the efflux rate of doxorubicin out of cells overexpressing MsbA in a concentration dependent manner (Fig. 11b).

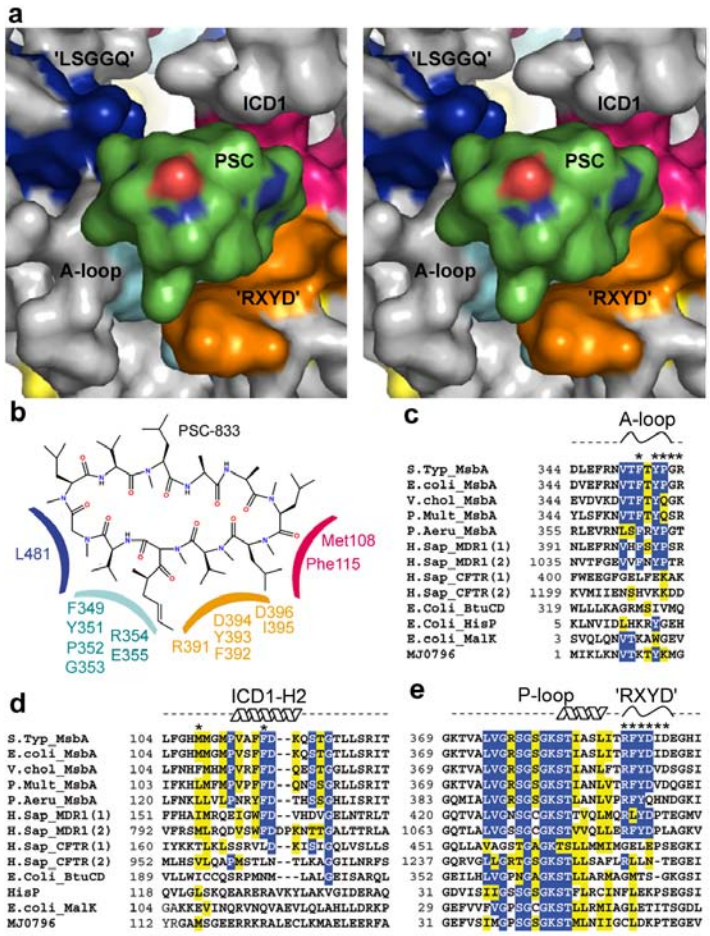


Figure 12. PSC binding site. **a.** Surface view of PSC binding pocket in stereo. The pocket is formed by conserved NBD elements: A-loop (cyan), 'RXYD' loop (orange), ICD1 (magenta) and the 'LSGGQ' signature motif (blue). PSC is shown as space-filled surface (green-carbon, red-oxygen, blue-nitrogen). **b.** Relationship of chemical representation of PSC to binding pocket elements. Sequence alignment of **c,** A-loop, **d.** ICD1, and **e.** P-loop and the 'RXYD' loop of MsbA, P-gp and other ABC transporters showing the sequence conservation of the PSC binding site; especially in the ABCB1/MsbA family.

Crystals of MsbA with PSC were grown as described[15] using detergent solubilized protein incubated with ATP, Mg^{2+}, sodium ortho-vanadate, Ra lipopolysaccharide (LPS), and PSC. The structure was determined by molecular replacement using the electron density from the MsbA·ADP·vanadate·Ra LPS crystal form for initial phases[22]. Electron density maps were improved by non-crystallographic symmetry averaging to a resolution of 4.5 Å. Although Ra LPS was present throughout the crystallization process, no density for LPS was observed in the PSC crystal form. A chemical model with good geometry was built with R_{cryst} of 28% and R_{free} of 39%.

MsbA is arranged as a homodimer composed of two interacting transmembrane domains (TMDs) and two nucleotide binding domains (NBDs) (Fig. 11d, 11e). Electron density for PSC was observed in a pocket formed between the ATP binding site and the second helix of intracellular domain 1 (ICD1-H2; residues 111-121) in each monomer (Fig. 11f). ICD1-H2 is formed between the cytoplasmic ends of TM2 and TM3. ICD1-H2

makes extensive contacts with conserved sequence motifs in the NBDs. PSC buries ~40% (~440Å^2) of its surface area by making van der Waals interactions with a sequence motif following the P-loop ('RXYD' loop), the ICD1, the 'LSGGQ' signature motif, and the ATP coordinating A-loop (Fig. 12a). A more detailed analysis shows that side chains within 6 Å of PSC include: loop 4 residues Arg391, Phe392, Tyr393, Asp394, Ile395 and Asp396; ICD1 residues Met108 and Phe115; 'LSGGQ' motif residue Leu481; and A-loop residues Phe349, Thr350, Tyr351, Pro352, Gly353, Arg354 and Glu355; (Fig. 12b). The ICD1, A-loop, and 'RXYD' loop that form the binding pocket of PSC are conserved in both MsbA and P-gp but vary considerably in other ABC transporters (Fig. 12c-12e).

Binding of PSC near the ATP binding sites causes significant structural changes in the TMDs. The binding of PSC causes a structural 'kink' in both the TM2 and TM3 helices at the TMD/ICD interface (Fig. 11d). This 'kink' pushes the second extracellular loop (EC2) away from the periplasmic end of TM1 and induces a significant bend in TM1 towards TM6. In the MsbA·ADP·V_i·LPS structure[22], TM1 and TM6 form a 'V' shaped opening with accessibility to the periplasmic side of the membrane (Fig 13a). In the PSC structure, this opening is closed as TM1 packs against TM6 (Fig. 13b). TM5 from one monomer also interacts more extensively with TM5 from the opposite monomer, disrupting accessibility to an internal chamber.

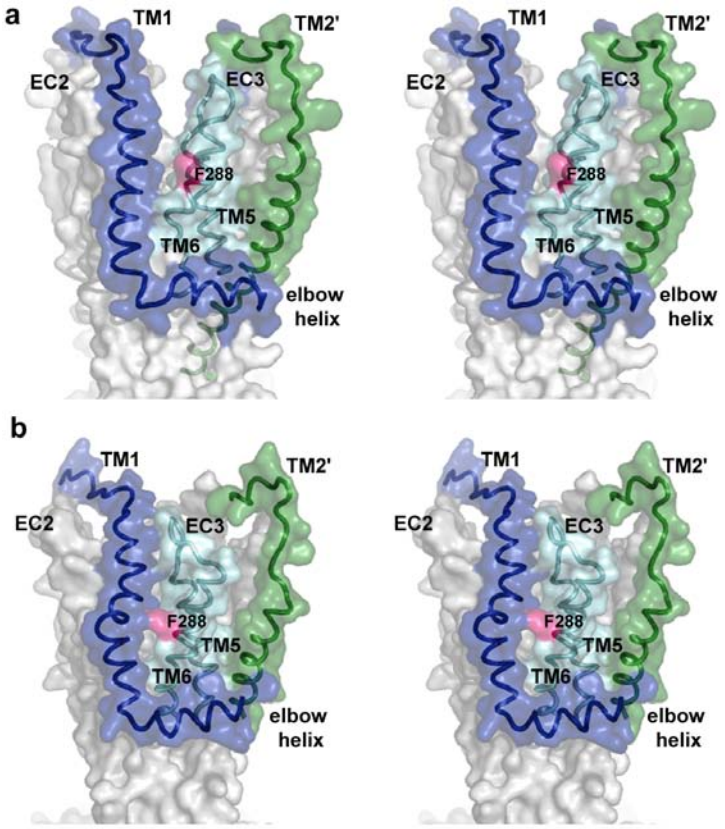


Figure 13. Transmembrane domain rearrangements upon PSC binding. **a.** Lipid-exposed face from the ADP·vanadate·LPS MsbA structure comprised of TM1 (blue), TM6/TM5 (cyan) from one monomer and TM2' (green) from the other monomer. The periplasmic exposed cleft is formed between TM1 and TM6/TM5. Phe288 is located on TM6 colored in magenta. **b.** Lipid-exposed face of MsbA in complex with PSC shows 'closure' of TM1-TM6/TM5 cleft with Phe288 buried in the interface as the extracellular loop 2 (EC2) moves away from TM1.

Rearrangements in the TMD helices provide insight into the mechanism of substrate transport and inhibition. Structural comparisons of the TMDs observed in MsbA·PSC·ADP·vanadate to MsbA·ADP·vanadate·Ra LPS and apo MsbA[23], reveals two grooves termed the *cis*- and *trans*- grooves, that are formed by helix-helix interactions formed by TM5-TM6 and bracketed on either side by $TM1_{cis}$ and $TM2_{trans}$ (*cis*- same monomer, *trans*-opposite monomer) (Fig. 14a). PSC binding disrupts transport by closing the periplasmic ends of these grooves. In the MsbA·Ra LPS structure, lipid binds to the extracellular loops centered on TM6. The absence of electron density for LPS in the PSC data, is likely a consequence of the conformational changes observed in these grooves. Biochemical studies of P-gp residues that alter the binding and transport profile of substrates caused by PSC and other cyclosporine derivatives map to both the *cis*-[24,25] and trans-[26] grooves. One such residue, Phe335 (human P-gp), maps to residue Phe288 in *S. typhimurium* MsbA (Supplementary Fig.1) and is implicated as a key residue in transport regulation[25] (Fig. 13a, 13b). Interestingly, this residue is located at the packing interface between TM1 and TM6, and may control the passage of substrate from the inner leaflet side of the *cis*- groove. We propose that mutations in this region will disrupt the close helix-helix packing interface required to mediate the transport in either groove. In addition, the outward-facing opening of the internal chamber is closed in this structure and suggests blockage of direct transport through an internal pathway.

Transport through an internal pathway has been previously speculated for ATP-independent ethidium bromide efflux by LmrA, a prokaryotic ABC transporter with functional homology to MsbA and P-gp[27].

The conformational changes caused by PSC reveal insight into the mechanism coupling ATP hydrolysis to substrate transport. A Gly185Val mutation in P-gp, significantly decreases transport of chemotherapeutic drugs and increases binding of photoreactive analogs of these compounds, while increasing basal ATPase activity[28]. This mutation uncouples drug stimulated ATP hydrolysis from drug transport. In MsbA, this residue maps to the strictly conserved Gly141 which is located at the site of the 'kink' in TM3 and is likely involved in the transmission of signal between the NBD and TMD (Fig. 11d). A second strictly conserved glycine (Gly94 in MsbA corresponding with Gly141 in P-gp) is also located at the 'kink' in TM2 and together with Gly141 may allow structural flexibility required for transmitting signal at the TMD/ICD interface. Based on this structure, PSC disrupts this signaling mechanism to decouple hydrolysis from transport.

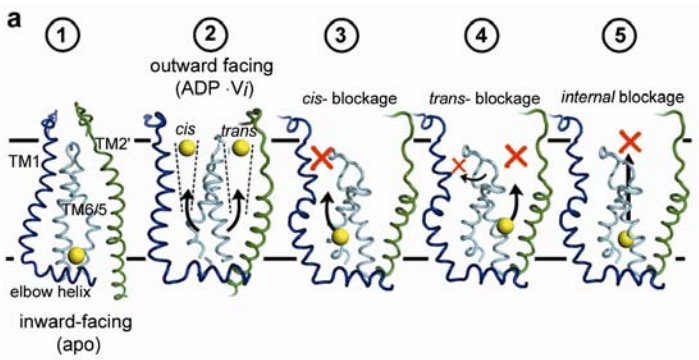


Figure 14. a. Proposed mechanism for PSC inhibition of drug transport. The elbow helix/TM1 (blue), TM6/TM5 (cyan) and TM2' (green) in the closed apo conformation of MsbA from *Vibrio cholera* (1). Substrate (yellow ball) initially binds to the inward facing side of membrane. Two grooves could facilitate transport (2). The *cis*-groove is formed between TM1 and TM6/TM5 from same monomer and the *trans*-groove is formed between TM6/TM5 and TM2 from opposite monomers. Binding of PSC causes a steric closure of the *cis*-groove (3), or alternatively, restricts movement of TM6/TM5 which in turn disallows opening of the periplasmic end of the *trans*-groove (4). Finally, reorientation of TM6/TM5 closes the outward-facing opening of the internal chamber, blocking substrate from exiting the transporter through a pathway between the monomers (5).

The PSC binding site resolved in this x-ray structure is well situated to disrupt the catalytic cycle of transport by interacting with the nucleotide binding pocket and ICD1. We propose that the ATPase activation/inhibition profile observed is a consequence of multiple binding events of PSC that alternately affect the integrity of the interactions between the nucleotide, ICD1s and conserved NBD motifs. At high concentrations PSC may bind as a substrate, stimulating ATPase activity by initiating the transport cycle. However, our doxorubicin transport data suggest that transport inhibition is independent of ATPase modulation. Therefore, transport is disrupted at all concentrations of PSC by decoupling ATP hydrolysis in the NBDs from transport pathways in the TMDs. Additional inhibitory effects likely occur at high concentrations of PSC through competitive binding to substrate binding sites.

The structural and functional analysis of MsbA with PSC provides a molecular structural basis for the modulation of ATPase activity and transport inhibition. PSC binds in a pocket near the the catalytic machinery uncoupling ATP hydrolysis from substrate transport. Other cyclic peptide compounds, such as dendroamides and patellamides, have been reported to be inhibitors of P-gp[29] and MsbA (data not shown) and may also have similar binding modes. In conclusion, this pocket represents a unique target in the development of novel cancer MDR efflux inhibitors as well as potent antibiotics that specifically target MsbA in Gram-negative pathogens such as *Yersinia pestis* and *Pseudomonas aeruginosa*.

Task 9. Determine high-resolution x-ray structure of estrogen receptor

We have discontinued this task to focus more on the inhibitors/modulators of MDR pumps taking the suggestion of the reviewer of the grant.

Task 10. Determine efficacy of compounds as clinically relevant compounds
We are initiating this task as we now have compounds and results from our *in vitro* functional assays.

Key Research Accomplishments

1. Production of six major classes of MDR inhibitor compounds suitable for click chemistry. We will be using these compounds as a starting basis to do click chemistry both *in vitro* and *in situ*.

2. Large scale production of integral membrane proteins human MDR1/mouse MDR3 and ABCG2 (Breast cancer resistance protein) for structural and functional analysis. This is done in collaboration Dr. Ina Urbatsch and the Chang laboratory. This is important for future structural and functional studies.

3. Development and the implementation of robust *in vivo* and *in vitro* functional studies to ascertain the inhibition of compounds. These assays are suitable for high-throughput screening.

4. Initiation of structural studies using x-ray crystallography with/without compounds for human MDR1/mouse MDR3 along with human ABCG2.

5. Initiated and working towards a close collaboration with Vera Donnenberg, whose lab will be assaying these compounds *in vivo* against tissue and human cell lines (moving towards Task 10 in this proposal).

Reportable Outcomes

Manuscript submitted and in progress:

Reyes, C. L., Ward, A., Yu, J., and Chang, G. Structural basis for inhibition of ABC transporter mediated efflux by the chemosensitizer PSC-833. Submitted to Nature.

Conclusions

We have combined structure, function, and chemistry as proposed for the design of new MDR inhibitors for breast cancer chemotherapy. We have developed hundreds of compounds and have discovered inhibition for six major classes of inhibitor. We have achieved high-level over expression of human MDR1/mouse MDR3 along with ABCG2, which is the breast cancer resistance protein. Our group is also doing extensive co-crystallization studies with known substrates/inhibitors as well as with our newly discovered compounds on both human MDR1/mouse MDR3 as well as ABCG2. We have recently determined the x-ray structure with the Novartis MDR reversal compound PSC-833, which is largely considered the gold standard in MDR inhibition. This structure has very wide impact on the clinical design of future MDR compounds. Our lab has also developed detailed functional assays (transport and ATPase assays) to ascertain the potency of inhibition and initiated close collaboration with the Donnenberg laboratory for the testing of these compounds in human MDR cancer cell lines. The discovery and the development of new compounds for the reversal of cancer MDR is well underway. This work will lead towards the development of drug compound that will reverse cancer multidrug resistance.

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Appendices

1. CV of PI

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The Scripps Research Institute	Asst Professor	1999	Molecular Biology
The Scripps Research Institute	Assoc. Professor	2006	Molecular Biology

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2001	Baxter Foundation Award
2001	Beckman Young Investigators Award
2000	**Presidential Early Career Award for Scientists and Engineers**
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1996	NIH Post-Doctoral Fellowship
1996	Saul Winegrad, M.D. Award for Outstanding Doctoral Dissertation
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Publications:

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Supporting Data

None.